Environmental Health Perspectives
Vol. 99, pp. 99-105, 1993

Does Aflatoxin Exposure in the United Kingdom Constitute a Cancer Risk?

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Although the aflatoxins were discovered more than 30 years ago, there is still considerable controversy surrounding their human health effects. Most countries have introduced legislation to control the level of aflatoxins in food, but it is not known if these permitted levels still pose a significant cancer risk. Furthermore, it is unlikely that all the sources of human aflatoxin exposure have been discovered, nor if the liver is the only, or indeed, major target organ for aflatoxin-induced cancer in man. In our laboratory we have used both immunological and HPLC methods to examine human DNA from a variety of tissues and organs to identify and quantify aflatoxin DNA-adducts. We have already detected aflatoxin B_1 (AFB_1)-DNA adducts in formalin-fixed tissue from an accute poisoning incident in Southeast Asia. Here we have examined human colon and rectum DNA from normal and tumorous tissue obtained from cancer patients and colon, liver, pancreas, breast, and cervix DNA from autopsy specimens. AFB_1-DNA adducts were detected in all tissue types examined and ranged from 0–60 adducts/10^6 nucleotides. Where sample size allowed, the adduct levels were confirmed by HPLC analysis. Tumor tissues tended to have higher adduct levels than normal tissue from the same individual, and levels generally increased with patient age. In samples analyzed by HPLC, the adducts present had the chromatographic properties of [8,9-dihydro-8-(N^5-formyl)-2′, 5′, 6′-triamino-4′-oxo-(N^5-pyramidyl)-9-hydroxy-aflatoxin B_1, the ring-opened form of the AFB_1-guanine adduct. The level of adducts observed in individuals from the UK were often in the same range as those seen in citizens of Third-World countries. AFB_1-DNA adduct values in this range, i.e., 1/10^6, have been reported to result in the development of tumors in rats and trouts dosed with AFB_1. This would indicate that aflatoxins may pose a carcinogenic risk to a variety of organs for the UK population and that there are either unknown sources of aflatoxin in our diet or that the present permitted level in food is too high.

Introduction

Aflatoxins are toxic secondary metabolites of several species of the Aspergillus molds, initially discovered as a result of the death of large numbers of turkeys and trout given contaminated feed in the 1950s. Since then a wealth of information has been amassed as to the chemical structure, metabolism, mutagenicity, and animal carcinogenicity of the aflatoxins and, in particular, the most potent animal carcinogen, aflatoxin B_1 (AFB_1). However, the possible role of aflatoxins in human carcinogenicity is still controversial (*1*). Epidemiological studies (*2–7*) have shown a correlation between aflatoxin exposure and primary hepatocellular carcinoma (PHC) incidence in several Third-World countries. These studies have been criticized for failing to take into account other confounding factors such as possible hepatitis B infection or exposure to pyrrolizidine alkaloids, selenium compounds, and alcohol (*1,8*). More recent studies have made attempts to compensate for such factors, some still finding a relationship between AFB_1 exposure and PHC and some not (*9–11*). Such studies illustrate that the multifactorial nature of human carcinogenesis makes it impossible to study the effect of a single compound in isolation. These studies also highlight how insensitive epidemiological studies of this nature are and show that it is insufficient to base exposure data only on analysis of food. The analysis of tissue DNA adduct levels, serum albumin adduct levels, and fecal and urinary aflatoxins should all be combined to give a more realistic picture of aflatoxin exposure.

DNA binding experiments, mutation assays, and RNA synthesis inhibition tests have also indicated that aflatoxin metabolism in man is more like that seen in refractory species such as the hamster than the susceptible rat (*8*). However, these studies have only used tissues from a few individuals and do not rule out the possibility that in the heterogeneous human population some individuals may be more susceptible than others, unlike an inbred species of rat. Also, a recent study on the biotransformation of AFB_1 by liver microsomes from a variety of species, using much lower substrate concentrations more representative of real exposure levels, found the proportion converted to AFB_1-8,9-epoxide (the ultimate carcinogen) increased at lower substrate concentrations in the case of rat and human microsomes (*12*). Furthermore, risk estimates of liver cancer due to aflatoxin exposure, extrapolated from animal and human studies concluded that, despite the Fischer rat being more sensitive than other species, risk estimates were all within a reasonable range and clearly within the same order of magnitude regardless of the species used to build the model (*13*).

A study in the United States compared liver, kidney, and colon cancer data for Southeastern rural and urban areas, on the assumption that aflatoxin exposure would be higher in the former

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due to the high consumption of corn grits and the local consumption of unmarketable peanuts. This study came to the conclusion that there was no correlation between aflatoxin exposure and cancer development (*8*). However, our previous findings (*14*; Harrison et al., in preparation) of aflatoxin adducted to liver DNA from UK and French residents not known to consume large quantities of either grits or peanuts, indicates that aflatoxin exposure in developed countries is probably much higher than previously thought, thus casting doubts as to the validity of the urban controls in the U.S. study. There have also been a number of clinical reports of high levels of aflatoxins in a number of patients including cancer patients from non-Third-World countries (*15–20*). While in no way proving that aflatoxin caused these cancers, these reports do support the hypothesis that aflatoxin exposure is not confined to the Third World.

Recent new sources of aflatoxin exposure have been identified in figs, yogurt, sunflower seeds, medicinal plants and even heroin. These sources are to be added to the list of previously identified sources of exposure now being consumed in increasing amounts in developed countries such as corn, rice, beans, nuts, and spices (*21–26*). All the above commodities and other, as yet unidentified, sources of aflatoxin exposure could therefore be contributing to a substantial aflatoxin burden in developed countries.

Many governments have therefore continued to class aflatoxins as probable human carcinogens (*27*), and 56 countries to date have imposed regulations to attempt to limit human exposure (*28*). The research therefore continues on a variety of fronts to establish what, if any, role aflatoxins play in human carcinogenesis. Ourselves and others (*14,29,30; Harrison et al., in* preparation) have examined human tissues for the presence of AFB_1 and its DNA adducts on the premise that this gives an indication of exposure and DNA damage potential. Other researchers have developed methods to analyze aflatoxins or adduct levels in a variety of body fluids, which would allow a much larger number of samples over a time course to be investigated than is possible by examining solid tissues (*31–35*). Such methodologies, especially in conjunction with the current hepatitis vaccination program, will allow the investigation of the contribution of these two possible culprits of human PHC in Third-World countries and the potential role of aflatoxins in cancer formation in developed countries.

AFB_1 is metabolised by liver microsomes to an 8-9 epoxide that can bind to glutathione to produce a less toxic metabolite, which can be transported around the body and excreted in the bile (*36*). However, the epoxide is also capable of binding to cellular proteins, RNA, and DNA. It is the binding of AFB_1 to DNA, to form DNA adducts (*37*), which has been related to tumor development in animals (*38*) and is believed to play a role in carcinogenesis. One possible mode of action of such adducts in cancer development has been indicated in contemporary studies of the *p53* tumor-suppressor gene. Mutational hotspots have been detected in the gene from human livers obtained from South Africa where aflatoxin exposure is known to be high (*39*).

Here we report on the surprising finding of aflatoxin adducted to DNA from a variety of tissues from UK residents who have not previously been considered to be exposed to significant amounts of aflatoxin. Using an immunological system composed of immunoaffinity chromatography purification and inhibition ELISA, described previously (*40*), we have detected AFB_1 bound to DNA in approximately 50% of samples analyzed from a variety of organs.

Adduct levels detected range from 0.03 to 60.5 adducts/10^6 nucleic acid bases, which represents up to tens of thousands of DNA adducts per cell. Similar levels of adducts have been correlated with liver tumor development in a variety of animal species (*40*).

Materials and Methods

DNA was purified from normal colon and rectum samples obtained during surgery at York District Hospital, predominantly from colon cancer patients. "Normal" colon, breast, pancreas, and liver samples were obtained from cadavers at autopsy at York District Hospital and The Whittington Hospital, London. Liver autopsy samples from Africa and Southeast Asia were provided by The Liverpool School of Tropical Medicine. All patient and sample details available are recorded in Table 1.

All samples were extracted and purified using the method of Gupta (*41*). Briefly, tissues were homogenized in 1% w/v SDS, digested with proteinase K (Sigma, Dorset, UK), extracted sequentially with phenol, phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1), digested with RNAse A and T1 (Sigma, Dorset, UK), and reextracted, as above. Once purified, samples were incubated in 15 mM Na_2CO_3/30 mM $NaHCO_3$, pH 9, for 2 hr to convert any AFB_1–DNA adducts present to the more stable imidazole ring opened (IRO) form (*42*). To enable the recovery of <90% of the AFB_1–DNA adducts present by immunoaffinity chromatography (*40*), DNA was subjected to acid hydrolysis (0.1 N HCl at 70 °C) for 20 min to release IRO AFB_1–guanine (*43*), and the pH was readjusted to neutral.

To increase the sensitivity of the AFB_1 adduct analysis and to remove any interfering compounds, all samples were passed through an immunoaffinity column containing an anti-AFB_1 monoclonal antibody (Biocode Ltd, York, UK). The column was prewashed with 15 mM NaCl/1.5 mM $Na_3C_6H_5O_7 \cdot 2H_2O$, pH 7.4, samples applied in the same buffer, and after further washing, any bound material was eluted with 100% methanol. Eluents were dried in a Univap centrifugal evaporator (Uniscience, London, UK), resuspended in 10 mM K_2HPO_4/150 mM NaCl, and boiled to denature any antibody eluted from the columns. Samples were quantified by comparison of inhibition levels in an aflatoxin inhibition ELISA with those obtained for a series of AFB_1–DNA standards prepared by peracid activation of AFB_1 (*43*). All samples were analyzed both neat and as 1/10 dilutions, in triplicate, on at least two separate occasions. Calf thymus DNA was used as a negative control to account for any nonspecific inhibition. We have previously established, using DNA extracted from rats treated with [^{3}H]AFB_1, that this system accurately quantifies adduct levels as confirmed by radioactivity measurement (data not shown).

Where sample size allowed, acid hydrolyzed DNA (pH 3) was analyzed by HPLC on an ODS2 C-18 25 × 0.3 cm reverse-phase column using a methanol gradient from 0–100% v/v over 30 min at 1 mL/min, controlled by a Gilson Anachem HPLC system. The column eluent was monitored at 362 nm UV and 470 nm fluorescence (excitation 360 nm). The peak areas and retention times were compared to a series of AFB_1–DNA standards synthesized by peracid activation, as above. Sample sizes of

Table 1. Patient details.[a]

Patient no.	Tissue	Type	Duke's stage	Age	Sex	Adducts/10^6 nucleotides	Autopsy samples: Patient no.	Tissue	Type	Duke's stage	Age	Sex	Adducts/10^6 nucleotides
1	Rectum	N	—	80	F	0	L:B:C:12	Liver, breast, cervix	N	—	32	F	1.02, 3.36, 0.48
1		T	C	80	F	0.67	L:13	Liver	N	—	72	M	0
2		N	—	—	F	0.26	L:14	Liver	N	—	92	F	0
2		T	—	—	F	10.26	L:16	Liver	N	—	69	M	0
3		N	—	66	F	4.2	L:P:23	Liver, pancreas	N	—	88	F	0, 0
3		T	B	66	F	2.9	L:C:26	Liver, cervix	N	—	82	F	0.59, 1.92
4		N	—	—	F	0	P:28	Pancreas	N	—	66	M	0.33
4		T	—	—	F	2.6	B:31	Breast	N	—	85	F	0
5		N	—	—	F	5.1	L:P:C:36	Liver, pancreas, cervix	N	—	89	F	1.35, 0.21, 4.9
5		T	—	—	F	5.3	L:P:39	Liver, pancreas	N	—	72	F	0, 0.33
6		T	—	—	M	0	P:40	Pancreas	N	—	76	M	0.29
26		T	B	82	F	0	L:P:C45	Liver, pancreas, cervix	N	—	66	F	0.23, 0.34, 0
27		N	—	64	M	0	P:B:54	Pancreas, breast	N	—	—	F	0.47, 0
27		T	B	64	M	0	L:57	Liver	N	—	72	F	0
7	Colon	N	—	—	M	3.07	L:P:59	Liver, pancreas	N	—	—	F	0, 0
8		N	—	—	—	0.19	L:P:B:60	Liver, pancreas, breast	N	—	—	F	0.43, 0, 0
9		N	—	—	—	3.07	B:70	Breast	N	—	—	F	0.43, 0, 0
17		N	B	71	M	0.72	L:P:37	Liver, pancreas	N	—	—	F	0.35, 0
18		N	—	52	F	2.6	P:38	Pancreas	N	—	—	M	0
18		T	C	52	F	1.0	L:56	Liver	N	—	—	F	0
19		N	B	71	M	0.04	L:67	Liver	N	—	—	M	1.29
30		T	A	71	F	0	P:72	Pancreas	N	—	—		0
32		T	B	52	M	0	F	Cervix	N	—	—	F	0
10	Sigmoid colon	N	—	84	M	0	414	Colon	N	—	—	—	0.62
10		T	B	84	M	0	404		N	—	—	—	0.24
11		N	—	—	M	0	420		N	—	—	—	0.17
11		T	—	—	M	4.1	406		N	—	—		0
12		N	—	74	F	2.2	409		N	—	—		0
13		N	—	—	F	0.41	413		N	—	—		0
14		N	—	—	M	2.07	418		N	—	—		0
15		N	—	—	F	0.41	407		N	—	—		0
16		N	B	75	F	0	P1[b]	Liver	—	—	—		1.8
20		T	B	65	M	6.1	P2[b]		—	—	—		19.8
23		N	C	60	F	0	P3[b]		—	—	—		1.5
24		N	—	64	F	0	P4[b]		—	—	—		0
24		T	B	64	F	56.9	P5[b]		—	—	—		0
21	Right colon	T	B	83	F	5.9	P6[b]		—	—	—		6.3
25		T	B	66	F	0	P7[b]		—	—	—		0
29		T	—	73	F	0	P8[b]		—	—	—		0
22	Transverse	N	—	—	F	0							
28	Colon	T	C	59	M	0							
31	Left colon	T	C	50	M	0							

[a]Patient details showing age, sex, tissue type (N, normal; T, tumor), Duke's classification (A, least invasive; D, most invasive), and AFB_1–DNA adducts/10^6 nucleotides, giving an indication of the invasiveness of tumors at the time of surgery; (—) unknown.
[b]Samples were from Africa and Southeast Asia.

Table 2. Comparison of the percentage of samples positive for the presence of AFB_1–DNA adducts from different tissues.

Tissue type	Number of samples analyzed	Number samples with AFB_1–DNA present	% of positive samples
Liver	46	30	64
Colon[a]	26	14	54
Control colon	10	4	40
Rectum	14	8	57
Pancreas	13	6	46
Breast	5	2	40
Cervix	5	3	60
Total	119	67	56

[a]From cancer patients.

300–1000 mg DNA were chromatographed and compared with standards of 100, 50, 10, 5 ng [8,9-dihydro-8-(N^5-formyl)-2′, 5′, 6′-triamino-4′-oxo-(N^5-pyramidyl)-9-hydroxyaflatoxin B_1 (FAPY). To establish if the imidazole ring-opened form was the adduct already present in the tissues, highly adducted samples were analyzed immediately after DNA extraction and after deliberate ring opening by incubation in mild alkali.

Results

Utilizing aflatoxin immunoaffinity chromatography combined with inhibition ELISA quantification for AFB_1–DNA adducts, we have detected adducts in all tissues types examined to date. Out of 117 samples analyzed, 65 had detectable levels of AFB_1–DNA adducts, representing from 40 to 65% of all samples tested, depending on the organ examined (Table 2). Many of the samples from UK residents had adduct levels within the same order of magnitude as samples from Southeast Asia and Africa (samples P1–P8), where aflatoxin exposure is known to be endemic (Fig. 1).

Samples that were analyzed by HPLC displayed a predominant retention peak at 16 min, identical to that seen for the synthetic IRO AFB_1–DNA (Fig. 2). Quantification of AFB_1–DNA adducts by the two methods gave comparable results (Table 3).

Comparison of HPLC peak retention times for samples analyzed immediately after DNA extraction and after alkali pretreatment indicate that the adducts present in the tissue were already in the ring-opened form. As it is the ring-opened form of the adduct that accumulates with chronic exposure (*44*), these results indicate that the levels of adducts detected in the UK residents are more likely to be due to chronic aflatoxin exposure than acute exposure prior to sample analysis. Where normal and tumor tissue was available from the same patient, the adduct levels in the tumor tissue tended to be higher than those seen in the normal tissue (Fig. 3).

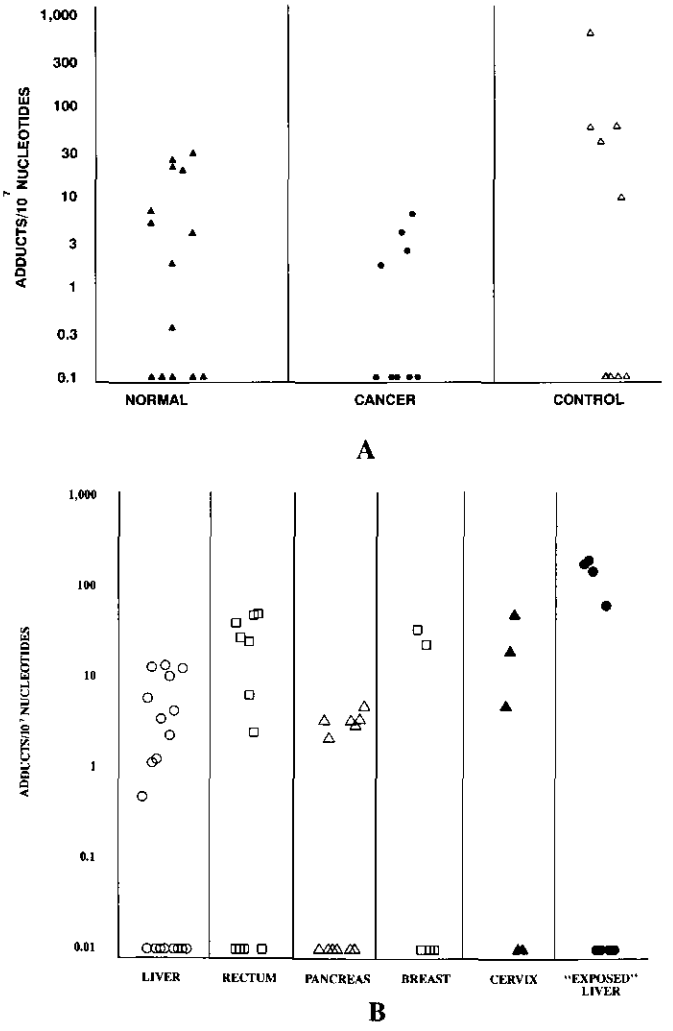

FIGURE 1. (*A*) Aflatoxin B_1–DNA adduct levels as measured by aflatoxin inhibition ELISA after immunoaffinity chromatography in normal and tumor tissue from cancer patients and "control" colon tissue from autopsy from autopsy samples. (*B*) Aflatoxin B_1–DNA adduct levels as measured by inhibition ELISA following immunoaffinity chromatography in a variety of organs obtained at autopsy including samples from "exposed" individuals from Southeast Asia.

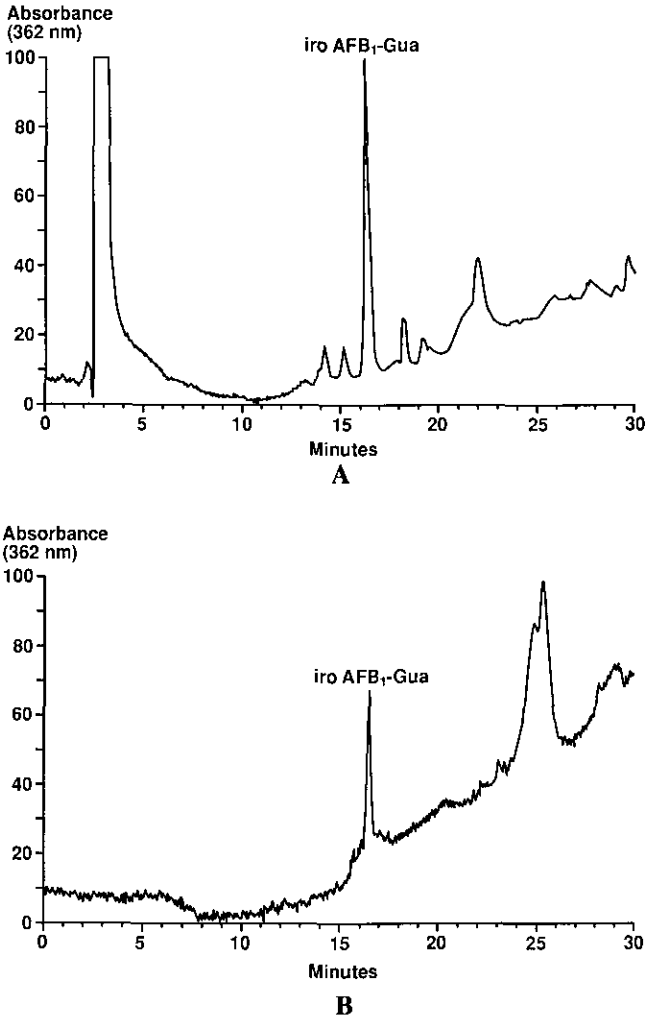

FIGURE 2. (*A*) HPLC trace of acid hydrolyzed imidazole ring-opened AFB_1–DNA synthesized by peracid activation of AFB_1. (*B*) HPLC trace of imidazole ring-opened acid-hydrolyzed human colon DNA. Colon DNA samples that had not been deliberately ring opened by incubation in alkali also gave the major retention peak at 16 min. Full-scale deflection = 0.1 absorbance units (FS) for UV and 0.01 relative fluorescence units (RFU) for fluorescence detection.

Table 3. Comparison of AFB_1–DNA adduct values in human colon as measured by competitive inhibition ELISA and HPLC.

	Adducts/10^6 nucleotides	
Sample no.	ELISA	HPLC
2 (Tumor)	10.60	20.30
12	2.20	2.20
14	2.20	2.20
14	2.01	2.20
15	0.14	0.70
24 (Tumor)	56.90	33.40

Discussion

The detection of AFB_1–DNA adducts in all tissue types examined to date parallels the situation with polyaromatic hydrocarbon (PAH)–DNA adducts in cigarette smokers. In the case of smokers, adducts were detected in all tissues examined, which

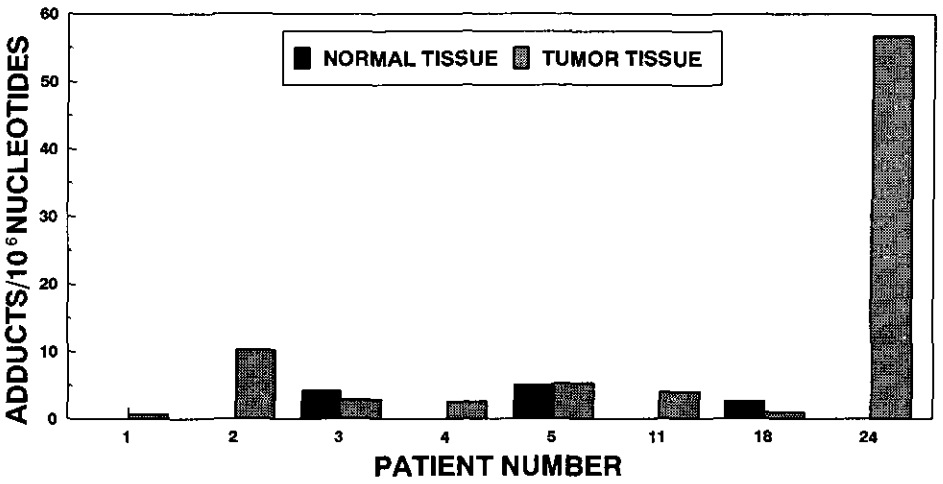

FIGURE 3. Aflatoxin B_1–DNA adducts in normal and tumorous colon tissues from the same patient, as measured by inhibition ELISA after immunoaffinity chromatography.

were also known target organs for cigarette-induced cancers. The levels of PAH–DNA adducts detected were also strongly correlated with the number of cigarettes smoked in a lifetime (*45,46*). The liver, colon, pancreas, bile ducts, and kidney have all been shown to be target organs for AFB_1-induced carcinogenesis in a variety of animals including nonhuman primates (*26,44,47,48*). Human colon explants have also been shown to be capable of metabolizing AFB_1 to produce DNA adducts (*49*), and a variety of human tissues are known to contain the P-450 isozyme thought to be responsible for AFB_1 activation (*50*; C. R. Wolf, personal communication). The levels of AFB_1–DNA adducts reported here are similar to those detected in human liver by ourselves and others (*14,40,51*). These adduct levels are of the same order of magnitude as those resulting in a 40–50% tumor incidence in rats and trout (*38,53,53*). Various reports indicate that there is a linear relationship between adduct level and tumor incidence in all species examined. The detection of AFB_1–DNA adducts in UK residents therefore may be indicative of persistent exposure to AFB_1, and this may be contributing to the cancer risk in a variety of organs.

The fact that the adducts detected (where sample size allowed) have been shown to be the ring-opened form indicates that there is a persistent, chronic exposure to aflatoxins in the UK. The average adduct levels seen here cannot be accounted for solely by the consumption of known sources of aflatoxin exposure: If AFB_1 metabolism in man in any way parallels that in the rat, the adduct levels seen would represent an exposure of 50 μg/kg (*52*), representing the consumption of some 350 kg of peanuts contaminated at 10 ppb. With the average per capita consumption of peanuts in the UK at 1.5 kg per year, there clearly must be other sources of aflatoxin exposure.

Utilizing the relationship between AFB_1-serum albumin adduct levels and organ DNA adduct levels (*53*) we have already begun an investigation as to the possible sources of aflatoxin exposure. By combining dietary questionnaires with the analysis of AFB_1-serum albumin adduct levels and the analysis of a wide variety of foods from local shops, we hope to establish the sources of the adduct levels reported here. We are also investigating the possibility of endogenous aflatoxin production by species of Aspergillus or other aflatoxin-producing fungi colonizing the gut, first by establishing in an animal model if such colonization is possible and then by investigating urinary and fecal total aflatoxin levels in patients on normal, synthetic, and fluid-only diets at the local hospital.

In five out of eight colon cancer cases, the AFB_1–DNA adduct levels in tumor tissue were higher than levels in normal tissue from the same patient (adducts not being detected in two of the patients and being equal in the third). These results are consistent with recent findings that show that the P-450 isozyme responsible for AFB_1 metabolism is elevated in certain liver and breast tumors, despite a general reduction of overall P-450 activity in tumors (*50*; C. R. Wolf, personal communication).

The sensitivity of different species of fish to AFB_1 induced tumors has been related to the difference in their ability to produce the reactive epoxide (*53*). The detection of AFB_1–DNA adducts in "normal" individuals and the lack of adducts in some cancer patients indicates that either *a*) some individuals lack the specific P-450 isozymes required to produce the epoxide; *b*) they have have high levels of the glutathione transferase isozyme required for detoxifiфcation; *c*) they have extremely efficient repair systems; *d*) they have never been exposed to AFB_1 or have adduct levels below the minimum detection level of this system (30 pg AFB_1).

In the last 30 years, much has been learned about the aflatoxins and their possible role in carcinogenesis. Their part in human cancer development is still fiercely debated and requires further investigation. Aflatoxins have predominantly been considered a potential risk for PHC development, as this is the primary target organ in the rat and because of the epidemiological links with PHC and AFB_1 exposure in the Third World. The results presented here indicate that the aflatoxins may in fact be a risk factor for cancer induction in a variety of organs in man, in the same manner as that of cigarette smoking. Other compounds, such as benzidine and 2-napthylamine, have produced tumors in rats at quite different sites to the primary target in man, and such might be the case with aflatoxins.

This manuscript was presented at the Conference on Biomonitoring and Susceptibility Markers in Human Cancer: Applications in Molecular Epidemiology and Risk Assessment that was held in Kailua-Kona, Hawaii, 26 October–1 November 1991.

We thank Mike Routledge and Nicola Froggatt, The Jack Birch Unit of Environmental Carcinogenesis, for their assistance; John Hopkinson, York District Hospital, for the patient details and Dukes' stage analysis; David Jenkins, The Wittington Hospital, London, for autopsy samples: and S. M. Peggy Maxwell, Liverpool School of Tropical Medicine, for samples from Africa and Southeast Asia. J.C.H. is funded by the UK Health and Saftey Executive. R.C.G. is partly funded by the EEC and York Against Cancer.

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